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Isolation and characterization of Xylanase producing strain of *Bacillus cereus* from soil

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ABSTRACT

Background and Objectives: Research on Xylanase has markedly increased due to its potential applications in pulping and bleaching processes using cellulose free preparations, textile processes, the enzymatic saccharification of lignocellulosic materials and waste treatment. The present study was aimed at isolation and characterization of xylan degrading strain of *Bacillus cereus* from soil for production of xylanase.

Materials and Methods: Twelve isolates were obtained from soil samples of different areas in the Rajshahi University campus and studied for detection of xylanase activity. One of the strains was identified as Bacillus cereus on the basis of the nucleotide sequence of the 16S rRNA gene which produces xylanase extracellularly. We purified xylanase to homogeneity by a combination of ammonium-sulphate precipitation, DEAE-sepharose, Phenyl-5PW and Hydroxyapatite column chromatography using culture supernatant.

Results: The SDS-PAGE gave a single band at 32 kDa. The optimum temperature and pH of the purified enzyme was 40^{o}C and 6.0, respectively. The xylanase hydrolyzed oat spelt xylan, birch wood xylan and beech wood xylan efficiently but showed no activity towards cellulose, CM-cellulose and Avicell pH 101.

Conclusion: Thus it was a true and neutral xylanase. The isolation of xylanase from *Bacillus cereus* is rare.

Keywords: Xylan, Xylanases, *Bacillus cereus.*

INTRODUCTION

Biodegradation of xylan, a component of the plant cell wall, is a complex process that requires the combined action of several enzymes, among which xylanase (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8) which cleaves internal linkages on the β-1,4-xylose backbone, play a key role (1). It has been shown that many kinds of bacteria and fungi hydrolyze β-1,4 xylan by the use of xylanolytic enzymes, such a β-1,4 xylanases, β-xylosidases, and esterases (2, 3). β-1,4 xylanases are the key enzymes that hydrolyze the backbone structure of β-1,4 xylans to initiate degradation of the complex polysaccharides by microorganisms. A number of β-1,4 xylanases have been purified from fungi and bacteria, and the genes encoding β-1,4 xylanases have been cloned and characterized. Several microorganisms produce multiple xylanases, implying a strategy for effective hydrolysis of β-1,4 xylan. Each of the enzymes may have a specialized function in the degradation of the complex polysaccharides and those specialized functions of individual xylanases may be useful for applications in human consumption, animal feed, and the paper industry (4, 5). Recently the interest in xylanases has markedly increased due to the potential applications in pulping and bleaching processes using cellulase free preparations, textile processes, the enzymatic saccharification of lignocellulosic materials and waste treatment (6).

Among the 12 strains isolated from the Rajshahi University campus, one was characterized according to Bergey's manual of systematic bacteriology (7) and was identified as *Bacillus*. We further characterized

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the strain on the basis of the nucleotide sequence of the 16S rRNA gene and the strain was found to be *Bacillus cereus*. There were many reports of xylanases from *Bacillus* sp. but xylanase from *Bacillus cereus* was rare. Probably, this is the first report of xylanase producing strain from *Bacillus cereus*. This study reports the isolation, purification and characterization of xylanase from *Bacillus cereus* obtained from soil.

MATERIALS AND METHODS

Bacillus cereus strain was isolated from soil samples, collected from Rajshahi University Campus and was maintained in medium I as follows: 0.5% oat xylan, 0.2% yeast extract, 0.25% NaCl, 1.5% KH_2PO_4, 3% NaH_2PO_4 0.5% NH_4Cl and 0.025% $MgSO_4.7H_2O$, pH 7.0.

Isolation and screening of the bacterium. Sample from soil in sterilized water was poured and spread onto agar plate A. These plates were incubated at 37°C for 2 days. The colonies on the plates were transferred onto agar plate B, which were again incubated at 37°C for 2 days. Colonies producing clear zone in the plate were selected.

Characterization of the bacterium. The morphological properties and taxonomic characteristics of the bacteria was studied by the methods in Bergey's manual of systematic bacteriology (7). The isolated bacteria was an aerobic, gram-positive, spore forming, rod-shaped organism.

Analysis of 16S rRNA gene. The sequence of 16S rRNA under accession number AY826631 was used to design primer for PCR and a partial 16S rRNA sequence was amplified by PCR using TaKaRa EX taq Hot Start Version (TAKARA BIO Inc.), as recommended by the manufacturer. Bacterial rRNA sequence was amplified using a pair of primers EU10F and UN1513R with 30 cycles of the following thermal program: 94°C for 30 sec, 55°C for 30 sec, and 72°C for 120 sec. The amplified DNA fragments were cloned with a TOPO TA cloning kit for sequencing (Invitrogen). The nucleotide sequence was determined with a Big Dye terminator Cycle sequencing Kit (Applied Biosystems) using universal rRNA-specific primers, M13 forward and reverse primer on an ABI PRISM 3100 DNA Sequencer (Applied Biosystems).

Xylanase purification. *Bacillus cereus* was aerobically grown at 37°C. for 48 h in a liquid medium as described above. The cells were separated by centrifugation at 12,000 × g for 10 min and used as crude enzyme. The purification of the xylanase was done at 4°C. The crude xylanase was precipitated with ammonium sulfate at a concentration corresponding to 80% saturation. The resultant precipitate was collected by centrifugation at 15,000×g for 20 min, dissolved in 50 mM sodium phosphate buffer (pH 7.0), and dialyzed against the same buffer and applied to the DEAE-sepharose column. The elution was done from 0-0.5 M NaCl. The xylanase active fraction was eluted at 0.25 M NaCl gradient. The active fractions from DEAE-sepharose column were combined, mixed with the same volume of 3 M ammonium sulfate and put onto Phenyl 5 PW column, which was previously equilibrated with 20 mM sodium phosphate buffer (pH 6.8) containing 1.5 M ammonium sulfate. Adsorbed proteins were eluted with a descending linear gradient of ammonium sulfate. Xylanase activities were eluted at 0.2 M ammonium sulfate. The active fraction from phenyl 5 PW was dialyzed against 5 mM sodium phosphate buffer of pH 7.0. The dialyzed enzyme solution was put onto a hydroxyapatite column previously equilibrated with the same buffer. The absorbed protein was eluted with a linear gradient of 5-100 mM sodium phosphate buffer (pH 7.0). The active fraction was eluted at 50 mM sodium phosphate buffer. Purity of xylanase in the fractions from column chromatography was analyzed by SDS-PAGE.

Protein measurement. Protein concentration was measured by the method of Lowary *et al.* (8). Bovine serum albumin was used as a standard.

Effect of temperature and pH on xylanase activity. The reaction mixture containing 0.9 ml of oat spelt xylan solution and 0.1 ml of the purified enzyme preparations was incubated at 30-80°C. The effect of pH on xylanase activity was studied in the following buffers; 50 mM acetate buffer of pH 4.0, 50 mm acetate buffer of pH 5.0, 50 mM sodium phosphate buffer of pH 6.0, 50 mM sodium phosphate buffer of pH 7.0, 50 mM Tris buffer of pH 8.0 and 50 mM glycine buffer of pH 9.0.

Xylanase activity assay. Xylanase activity was assayed using 1% solution of oat spelt xylan as the substrate as described by Baily *et al* (9) and the amount of reducing sugars released was determined by the dinitrosalicylic acid method (10). One unit of

enzyme activity was defined as 1 mM xylose equivalent produced per minute under the given conditions.

RESULTS AND DISCUSSION

Isolation and screening of the bacterium. For the preliminary experiment of this study, bacteria samples were collected from soil. Xylanolytic clear and transparent zone (on the xylan agar plate) producing bacterial strains were collected and incubated at 37°C for 48 h. After collection of bacterial strains by pure culture technique, the colonies were screened by the staining method, microscopic examination, and oxidase and catalase test. Finally we tried to verify one of the strains by sequencing the 16S rRNA gene described in the materials and methods section. As a result, the 16S rRNA gene of the strain revealed 94% identity with *Bacillus cereus* (Table 1).

Purification of xylanase. Results of xylanase purification from *Bacillus cereus* are summarized in table 2. The purication of xylanase was done by four steps; ammonium-sulphate precipitation, DEAE-sepharose, Phenyl-5PW and Hydroxyapatite column chromatography as described above. The xylanase was purified 16.3 fold, with a final yield of 13.4 %. The specific activity of the purified enzyme was 19.8 mM/min/mg. Analysis of the purified enzyme by SDS-PAGE revealed a single band with a molecular mass of 31 kDa as determined by SDS-PAGE (Fig. 1). Recently, the cloning and characterization of xylanase A from the strain *Bacillus* sp. BP-7 was done and reported by Gallardo *et al.* (11). They found the low molecular weight xylanases of family 11 and it was 27 kDa. Comparatively the xylanase from *Bacillus cereus* is little higher molecular weight than the xylanase from *Bacillus* sp. BP-7.

Optimum temperature and pH of xylanase. The effect of temperature on activity of xylanase against oat spelt xylan was examined in the temperature range of 30-80°C. The xylanase showed maximum activity at 40°C (Fig. 2). The xylanase exhibited greatest activity in the pH range of 5-8, having maximum

Table 1. The Nucleotied sequences of the partial 16SrRNA gene of the isolate identifiedas *B.cereus.*

16S rRNA Gene Sequence
AGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGC
GAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGCACGGGTGAGTAACACGTGGGTA
ACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGA
ACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATT
AGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCG
GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAA
TGGACGAAAGTCTGACGGAGCAAC

Table 2. Purification of xylanase from *Bacillus cereus*.

Steps	Volume (ml)	Protein (mg)	Activity (mmol./min)	Recovery (%)	Specific activity (mmol/min/mg)	Purification (fold)
Culture	20	512	623	100	1.21	1
Ammnium	4	220	323	51.8	1.46	1.21
DEAE	6	53	127	20.3	2.39	1.98
Phenyl	5	13.5	97	15.5	7.18	5.93
Hydroxy-apatite	4	4.24	84	13.4	19.8	16.3

value at 6.0 (Fig. 3). The optimum temperature of purified xylanase in this study is similar to the optimum temperature of alkaline xylanase from *Bacillus pumilus* (12). The optimum pH is similar to high molecular weight xylanase from *Aeromonas caviae* W-61 reported by Roy *et al*. (13).

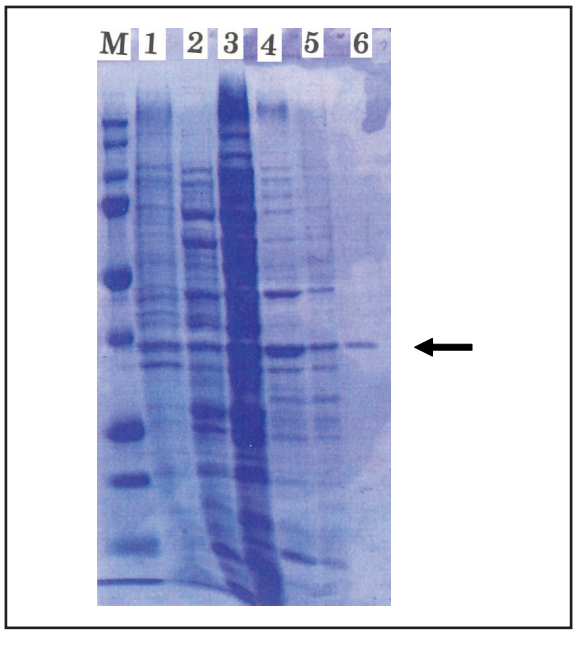

Fig. 1. SDS-PAGE analysis of xylanase purification from *Bacillus cereus* Lane. M, protein standard marker (molecular mass from top: 200kDa, 150 kDa, 100 kDa, 75 kDa, 37 kDa and 25 kDa); 1, culture supernatant; 2, ammonium sulphate precipitant; 3, DEAE-sepharose; 4, Phenyl 5, PW and 6, Hydroxy-apatite. Arrow indicates the xylanase enzyme.

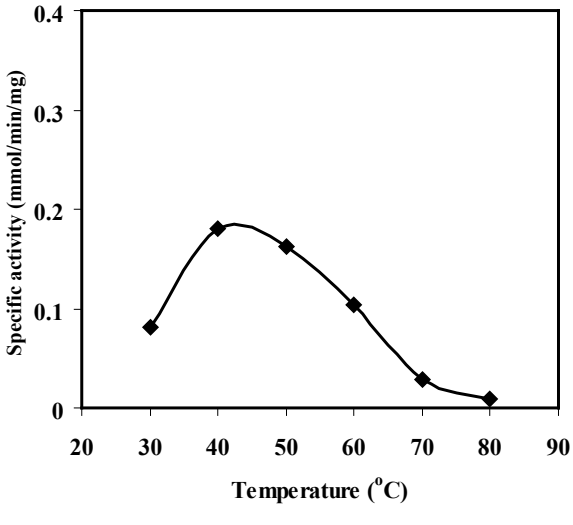

Fig. 2. Effect of temperature on purified anzyme.

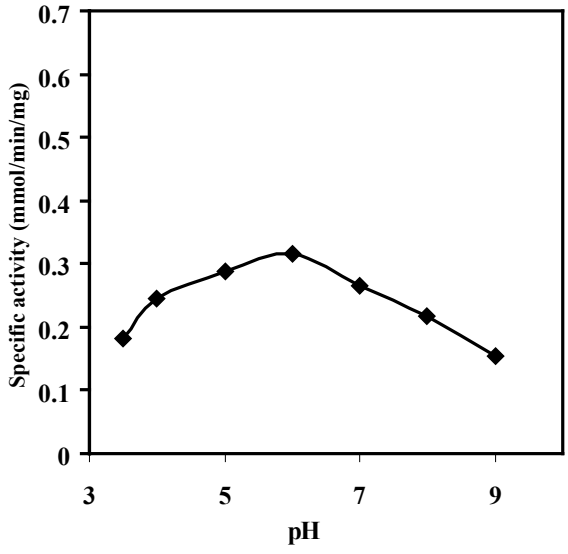

Fig. 3. Effect of pH on purified enzyme.

Substrate specificity of xylanase. The substrate specificity of purified xylanase was studied using various polysaccharides as substrates (Table 3). From the table, it was shown that the xylanase strongly hydrolyzed oat spelt xylan, birch wood xylan and beech wood xylan. However, the xylanase could not hydrolyze cellulose, CM-cellulose and Avicell pH 101**.** These properties were found in the xylanase 1, 2 and 3 reported previously (14, 15) and other xylanases from *Bacillus pumillus* (16) and *Bacillus subtilis* (17). Thus it was a true xylanase.

The isolation of xylanase from *Bacillus cereus* is rare. Its maximum activity was found at 40°C and pH 6.0. So it may be considered as a neutral xylanase. It should be interesting to further study the molecular biology and structural features of this type of xylanase.

Table 3. Substrate specificity of xylanase from *Bacillus cereus.*

Substrate	Relative activity (%)
Oat spelt xylan	100
Birch wood xylan	94
Beech wood xylan	86
Cellulose	0
CM-cellulose	0
Avicell pH 101	0

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